Biochemical studies of the higher level systematics of birds

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INTRODUCTION

The use of biochemical methods in avian systematics has a significantly long history, with early reports involving immunology dating back almost 100 years (e.g. Nuttall 1904); consequently, these methods are nearly as old as the *Bulletin* of the British Ornithologists' Club.

Nevertheless, the quantity of early research was minimal and the results not particularly influential until a major increase in such studies began in the 1960s. By that time the technique of protein electrophoresis had been developed sufficiently to allow rapid surveys of samples of blood and egg-white, and it quickly replaced the earlier use of serological and immunological techniques (e.g. the research programme of workers such as Erhardt 1930, Irwin & Miller 1961, Mainardi 1963 and Stallcup 1961). Rapid developments in medicine and molecular genetics were quickly integrated into research programmes in biochemical systematics. During the same period of time, advances in analytical methods, along with philosophical insights into the nature of phylogenetic inferences, had resulted in rapid changes in systematic practice. Thus, the advances in biochemical technology and analysis used in avian taxonomy during the past 30 years, reviewed here, have been remarkable. The earlier, largely immunological work on avian systematics was reviewed previously by Sibley *et al.* (1974).

In considering the achievements of biochemical systematics, important distinctions arise concerning the taxonomic level of the problem. Microtaxonomic or microevolutionary studies are inquiries which are aimed at elucidating the genetic structure of populations and problems of the origin of species; whereas macrotaxonomy is the study of the relatedness and phylogeny of species and higher taxa. Until now, the success of molecular methods at these 2 levels has differed considerably. The quality and impact of biochemical studies of macrotaxonomy are the subjects of this review. Biochemical studies of microevolution in birds were the subject of earlier reports (Barrowclough 1983, Evans 1987).

THE ELECTROPHORESIS ERA

General proteins

Some electrophoretic experiments on avian proteins were reported as early as the 1930s (Landsteiner *et al.* 1938); however, McCabe & Deutsch (1952) reported on comparative electrophoretic mobility of egg-white proteins from 37 species representing several orders of birds, and it was this work that led Sibley, his graduate students and post-doctoral associates to begin a major research programme in electrophoresis, principally

aimed at elucidating higher level avian relationships. The work involved first paper, and later starch-gel electrophoresis of egg-white proteins followed by treatment with a general protein stain. Additional studies involved haemoglobins, and blood plasma proteins, eye lens proteins, and feather keratins. The utility of the technique lies in the detection of mobility differences among proteins based on their electrical charge and the shape of the molecule; thus, similarities and differences among taxa are documented. Two major monographs (Sibley 1970, Sibley & Ahlquist 1972) summarized many of these results.

Statements about the Sturnidae are typical of the results reported in these works. For example, egg-white electrophoretic patterns of 10 species of starlings in 7 genera are cited in the passerine volume; the patterns of *Sturnus* and *Lamprotornis* were found to differ in a major component protein and those of *Sturnus* and the woodswallow *Artamus* appeared to match well. Thus, Sibley was able to conclude, among other things, that the first 2 genera may not be closely related and that the Artamidae are one of the most likely relatives of the Sturnidae.

Techniques for separation of proteins became more sophisticated in the latter 1960s and early 1970s and, at least in Sibley's laboratory, starch-gel methods were replaced by acrylamide gels and then isoelectric focusing in acrylamide. The amount of energy, resources and talent that went into this research, in a number of laboratories, over 2 decades was enormous; the quantity of data produced was voluminous. Moreover, the problems addressed were of genuine taxonomic interest. Unfortunately, however, it is clear in retrospect that this research programme produced few lasting accomplishments. This was due to 2 factors: the absence of quantitative methods of analysis and insufficient informative characters. Neither of these problems was understood by the systematics community at the time of the work, nor was the failure peculiar to one research programme.

It is now generally recognized that in order to infer a phylogeny, it is necessary to use cladistic or other phylogenetic methods that can distinguish between derived and primitive similarity (e.g. Wiley 1981). This recognition only became widespread in the 1970s, after much of the general protein work had been eclipsed by newer techniques. In principle, phylogenetic methods could have been applied to egg-white and other studies of proteins. For example, if homology of specific proteins on the gels could be established from species to species—a serious problem with total protein staining methods—alternate mobilities could be treated as alternate character states and analysed in a phylogenetic framework. In fact, this line of reasoning later was pursued by workers such as Brush & Witt (1983) and Knox (1980).

For example, in a study of the relationships of several species of Pelecaniform birds, Brush & Witt determined the electrophoretic mobility of feather keratins under several pH conditions; they computed genetic distances among the taxa, based on the sharing of bands among species, using general protein staining. Phylogenetic trees were computed based on the genetic distances. The trees were generally similar in that congeneric species tended to stay together, but the precise branching patterns depended on both the pH of the electrophoretic experiments and

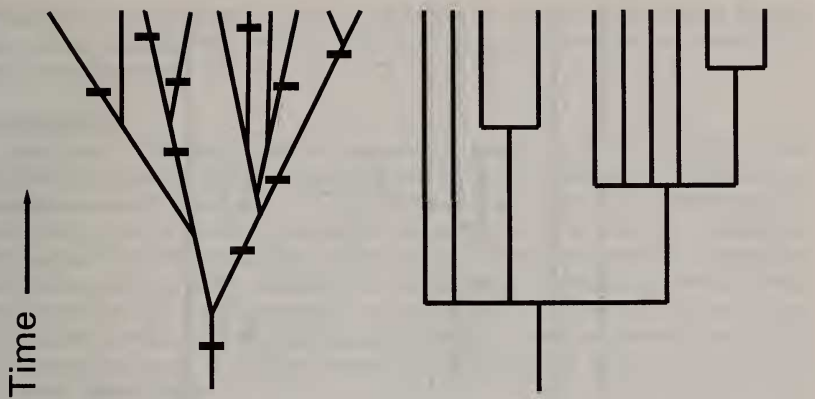

Figure 1. The details of evolutionary branching patterns cannot be recovered given too few characters or when branching events are too close in time. Left: Hypothetical evolutionary tree for 10 species with 10 character changes, randomly distributed in time, indicated by bars. Right: Maximally informative tree that can be inferred from character data shown on left.

the algorithm used to infer the tree. Such results indicate the dependence of pattern identity on pH and the sensitivity of tree topology to assumptions in the algorithms about constancy of rates of evolution.

The second serious problem with the general protein approach was the number of characters available for analysis. In a starch-gel, egg-white studies might indicate a half dozen or so easily visualized proteins. For avian haemoglobins, the number is less. If one's objective is to work out a fully resolved phylogeny for the taxa of interest (i.e. no nodes of the tree generating more than 2 lineages), then there must be at least one mobility change in one character between every pair of nodes of the actual tree (Fig. 1). Consequently, there must be more total character states in the data set than there are taxa in the study. Moreover, if the amount of evolutionary time between nodes of the actual tree was short for some of the branches, then the probability of a character state change for that branch is also small. Therefore, a large number of characters are necessary for there to be a significant chance of a change occurring along the short branches (e.g. see Lanyon 1988). For electrophoresis of egg-white, the number of potential character states is insufficient to produce a fully resolved phylogenetic tree for more than a few taxa (Table 1). With isoelectric focusing, the number of distinguishable proteins and mobility states increases, but then homology problems also are accentuated.

Once these 2 major problems are acknowledged, it is apparent that either many more characters must be made available in order to attack the major problems of higher level systematics (for example, the relationships of the families of birds) or one must be content with lesser goals: identifying clades rather than working out fully resolved trees or allocating a taxon of dubious affinities to one of several suspected groups. It was at this reduced level of resolution that egg-white studies achieved some limited success. For example, Sibley (1968) was able to show that

TABLE 1

Approximate time, in PhD project equivalents during decade when technique was in vogue, to solve major problems in avian systematics using various biochemical techniques

Nature of Problem	General Protein Electrophoresis	Allozyme Electrophoresis	Microcomplement Fixation	Peptide Mapping	Amino Acid Sequencing	DNA-DNA Hybridization	Restriction Enzymes	DNA Sequencing
Allocate a problematical species to 1 of 5 families:	1	1	1	1	5	1	1	1
Resolve relationships of 25 species in a family:	IC	1 (IC?)	1	1 (IC?)	25	1	1	1
Resolve relationships among single representatives of each of 175 families of birds:	IC	IC	50	IC	175	50	7 (IC?)	7
Complexity of technique:	N	N	N^2	N	N	N^2	N	N

Notes. IC = Technique not capable of resolving problem due to insufficient characters.
N = number of taxa.

Zeledonia belonged somewhere in the New World 9-primaried oscine assemblage rather than with either the wrens or thrushes, as previously had been thought.

Allozymes

Lewontin & Hubby (1966) introduced the technique of electrophoresis followed by specific enzyme staining to a general audience of evolutionary biologists and systematists. This represented a major breakthrough: it made many more characters available than previously and was a relatively inexpensive technique. The ability to examine the mobility states of single enzymes greatly reduced the homology problem present in total protein staining. Furthermore, the number of different enzyme stains developed for medical clinicians and human geneticists meant that 30 or 40 characters were available, some of which might show 3, 4 or more different mobilities.

By the late 1970s and early 1980s a substantial number of workers in avian systematics were using allozyme techniques. Initially much of this research involved somewhat higher level systematics, for example, working out the relationships of species within genera and genera within families (e.g. Barrowclough & Corbin 1978, Avise *et al.* 1982, Gutiérrez *et al.* 1983). However, the quantity of this work was soon superseded by studies of intraspecific variation, for example, hybrid zones (e.g. Barrowclough 1980, Corbin *et al.* 1979), differentiation among introduced populations (e.g. Baker & Moeed 1987), differentiation among vocal dialects (e.g. Baker 1982), and variation among variable taxa that might represent either species or subspecies (e.g. Zink 1988, Johnson & Marten 1988). These studies of intraspecific variation did have major impact on avian systematics in the broad sense: in North America they led to changes in opinion about species status of a number of taxa (e.g. see Johnson & Marten 1988). More generally, they led to a general interest in avian evolutionary genetics and a more quantitative understanding of the population genetic structure of birds (Rockwell & Barrowclough 1987). The impact of the higher level studies, however, was considerably less.

Early on in the allozyme research enterprise it became clear that there was considerable heterogeneity in the degree of variability and rates of differentiation among the various genetic loci routinely analysed with allozyme electrophoresis (e.g see Evans 1987). Thus, although it might appear that 40 loci and 100 or more alleles were available for studies of higher level relationships, in fact this was a major overestimate. Some loci, for example the malate dehydrogenases, were so uniform that little variation was detected at the level of orders of birds; such loci were consequently of little value for studies of phylogeny within families or genera. Other loci, for example the non-specific esterases and adenosine deaminase, varied so much that they were useless for studies much above the species level—so many alleles were present that results were dominated by alleles unique to each taxon (autopomorphy) and convergences (homoplasy) rather than shared derived mobility states (synapomorphy). Consequently, at any given taxonomic level, the number of informative character states was often quite small and true cladistic studies produced

polychotomies—unresolved phylogenies—because there were no character states defining many of the possible nodes. This was the same limitation found in the earlier studies involving general protein staining.

A second major problem with many allozyme studies at higher levels was a general failure of investigators to take on real taxonomic problems. In the late 1970s, when the techniques first became available, a smattering of general studies was perhaps justified in order to determine the usefulness of the technique, the level at which it was useful, etc. Unfortunately, however, the allozyme work never matured to the point where workers consistently investigated problems such as phylogeny of all or even the major part of a taxon. Thus, one might find a study of a few thrushes representing a small fraction of a taxonomic problem. Studies such as Gerwin & Zink's (1989) phylogeny of 8 of the 9 species of hummingbirds in the genus *Heliodoxa* were in a minority. At higher taxonomic levels even less was done; thus, the taxonomic awareness and intensity found in Sibley's earlier work with general protein staining was not generally present in researchers using the newer technique. In the cases in which researchers pushed the technique in useful directions, some interesting results were produced. Kitto & Wilson (1966), for example, found that one allele at the mitochondrial MDH locus defined the order Charadriiformes and a second allele identified the major clade of swifts plus hummingbirds. Likewise, Matson (1989) found the expression of a testes-specific lactate dehydrogenase that was a synapomorphy for Columbiformes. Such limited, but useful, results were, in retrospect, about all that should have been expected from the technique at the familial and ordinal levels.

Allozyme electrophoresis had its lasting impact at lower taxonomic levels. First, the potential at higher levels was not widely appreciated and pursued and, second, given that the number of potential character states was always destined to be small at any taxonomic level, fully resolved phylogenetic trees were not really a possibility. The few interesting results that were achieved at higher levels did not have major influence on practical systematics; the results were often not new, the taxonomic sampling was too restricted, or the results were ambiguous. For example, Johnson *et al.* (1988) examined most of the species in the family Vireonidae using allozyme electrophoresis. Their results indicated long, separate, evolutionary histories for several lineages of these birds; however, the detailed phylogenetic relationships inferred for the taxa varied depending upon the algorithm used to analyze the data. Thus, many of the details necessary to produce a new taxonomic treatment of the family remained ambiguous.

OTHER PROTEIN TECHNIQUES

Microcomplement fixation

The immunological response of an organism to an antigen is based on many aspects of both the composition and conformational structure of the molecule *in vivo*. Thus, although the biochemical nature of the reaction itself is still the subject of intense investigation, it is nevertheless clear that more information is being assayed than just charge and size, as in electrophoresis. Less information is available than in direct amino acid

sequencing of the protein, but the cost is a fraction of that technique. Consequently, microcomplement fixation was used in a few labs for a decade or so in the late 1970s and the 1980s. This method allowed one to obtain a quantitative measure of the similarity of proteins based on the immunological response of the antibodies produced against a protein from one taxon on the homologous protein of the other taxon.

The major limitation of the method is that the technique of necessity yields information on the overall immunological distance between 2 organisms for a given protein; individual character information is not available. Thus, any phylogenetic analysis would require distance methods. These are less reliable than character methods because of their inability to identify specific apomorphies and homoplasies and, unless one assumes the protein evolves in a clocklike fashion, at the very least immunological experiments must be performed on all combinations of taxa; that is, a complete matrix of information is required. This makes the technique more and more expensive as the number of taxa, N, increases because the number of requisite experiments becomes of the order of N^2. Consequently, the technique would be most useful for working out relationships of a few species or allocating a species of unknown affinities to one of two or three higher taxa. For an investigator working out the relationships of approximately 175 avian families, other techniques would make more economic sense and ultimately involve less time.

Allan Wilson and his associates worked with the technique for approximately 15 years. In this period of time they used 4 major proteins (albumin, lysozyme, ovalbumin and transferrin) and addressed problems at several levels in a number of avian groups. Of particular interest to Wilson were the galliform birds; in part this was due to the availability of substantial quantities of proteins for birds frequently kept in aviaries. However, in spite of the effort directed at the problem, the results that could be obtained were restricted by the necessity to do reciprocal experiments, obtain a complete $N \times N$ matrix of data, and confirm results with alternate proteins. For example, Prager & Wilson (1976) reported on a study of 24 species from 4 families of galliforms and 1 and 2 representatives each of 13 other orders of birds. The resulting phylogenetic tree (based on a distance analysis) was surprisingly similar to some recent results of Sibley & Ahlquist (1990); nevertheless, the sparsity of the taxonomic sampling, the publication in a non-ornithological journal, and the inconsistencies with the then current ideas about relationships, led to the paper having little impact on avian systematic thought.

Peptide mapping

Electrophoretic experiments are capable of separating homologous proteins that differ in charge, isoelectric point and, to a lesser extent, shape. Nevertheless, it was recognised that 2 proteins with identical electrophoretic mobility could still vary in amino acid sequence. Before amino acid sequencing became available, information on sequence differences could only be obtained by indirect methods. One briefly popular technique was to cleave a purified protein into peptides with enzymes having highly specific activity (e.g. trypsin) and then compare the resulting peptides using electrophoresis and chromatography. Although a

substantial number of characters can be obtained per protein with this method (20 or more for ovalbumins), establishing homology of the individual peptides can be a difficult problem. In addition, the technique is time-consuming and expensive; it never became very popular. Of the little published work, the best such study was Corbin's (1968) work on pigeons of the genus *Columba*; however, that analysis predates the adoption of modern phylogenetic methods. Thus, his results were summarized in statements concerning the degree of similarity of taxa rather than as a phylogenetic tree.

Peptide mapping, like all the techniques listed thus far, involves an indirect method for revealing character differences resulting from amino acid sequence differences. By the mid-1960s it had become possible, albeit laborious, to obtain the actual amino acid sequence.

Amino acid sequencing

A large protein may consist of a string of as many as several hundred amino acids, each of which can take on, in theory, one of 20 states. Thus, the sequence data for just a few proteins has the potential to yield a vast quantity of systematic data. Moreover, by the time amino acid sequences began to appear in numbers in the mid-1970s, numerical phylogenetic techniques were also becoming available. Consequently, by picking proteins evolving at an appropriate rate, investigators might have used this technique to solve most of the major problems in higher level avian systematics. Many researchers realized this, but the technique never became labour or cost effective. Allan Wilson mentioned (pers. comm.) in 1977 that he thought sequencing the lysozyme of a single species was a good Ph.D. project. Thus, given approximately 175 families of birds and 2000 genera, the higher level systematics of birds might have been worked out in a few generations of researchers (see Table 1).

In fact, however, amino acid sequences were worked out only for a small number of taxa for a few proteins because the Wilson lab at the University of California at Berkeley was the sole major proponent of the technique among avian systematists. For example, one report from Wilson's lab (Jolles *et al.* 1979), reported lysozyme sequences for 9 species of birds; a chachalaca (Cracidae) was found to be quite distant in its sequence from other galliforms, including a guineafowl. Biochemical physiologists also sequenced a number of avian globin genes, but those data were never synthesized and brought to the attention of avian workers. The amino acid sequence data eventually produced were too few to have a real effect on avian systematics.

THE DNA ERA

All protein methods, including amino-acid sequencing, are unable to reveal some potentially useful character state data because proteins are translated from DNA sequences that contain more information than do the corresponding amino acid sequences. For example, the 2 DNA sequences TTA and CTG both code for the same amino acid, leucine. If 2 taxa of birds had these alternate sequences, protein techniques would fail

to detect the differences. In theory, then, studies of DNA offer the potential for more characters, hence a greater chance of finding state changes along short branches, thus more resolution, etc. All protein methods can be viewed, in fact, as indirect attempts to get at DNA sequence information. As soon as the relationship between DNA sequences and protein coding was understood in the 1950s, it was realized that DNA analysis would be the ultimate tool of molecular systematics. However, DNA sequencing was preceeded by less direct techniques for developing this rich source of information.

DNA-DNA hybridization

Shields & Strauss (1975) first reported on the application of a method for comparing the overall DNAs of species in their study of some New World finches. In this method an index to the similarity of the DNAs of a pair of species is found by monitoring the melting temperature of hybrid molecules of DNA formed in the laboratory. In general the more similar 2 DNA sequences, the higher the melting point; this can be precisely measured under controlled conditions. A general review of the technique can be found in Sibley & Ahlquist (1990). The approach was adopted by Sibley and his colleagues in the early 1970s and a major research programme undertaken that has ultimately involved comparisons of thousands of individuals of hundreds of species.

The results of this research have been quite controversial, largely because of concerns about the method of analysis of the data. Besides problems of data reduction peculiar to the Sibley & Ahlquist laboratory (Lanyon 1992), the method itself inevitably produces only a measure of the overall distance between 2 taxa. Thus, there are no character data to analyze; consequently, methods of phylogenetic analysis that are free of assumptions about evolutionary rates ought to be used and these necessitate a complete matrix of intertaxon distances. Once again this causes the amount of work involved to increase as the square of the number of taxa. For practical reasons, then, DNA-DNA hybridization studies must involve a small number of taxa or involve dubious assumptions about evolutionary rates. The advantage of the technique was that it does examine DNA; hence in the 1980s it represented a novel dataset, and it involves a large part of the total genome, not just a single gene or class of genes (e.g. enzymes). However, contrary to statements of some proponents, the latter advantage does not entirely vitiate problems of rate differences. It was argued (e.g. Sibley & Ahlquist 1983) that, because a very large number of genes were being analyzed, rate differences among genes would average out to a grand mean and produce an overall constant rate of evolutionary change. This would make the data easier to collect and analyze because a complete data matrix is not always essential for clocklike distances. It is true that the law of large numbers works to the technique's advantage for one of the sources of variation—average differences in evolutionary rates among genes within individuals. However, other sources of variation are not affected by this averaging; these include changes in rates of substitution across the entire genome due to: 1.) demographic events, such as fluctuating population sizes; 2.) relative efficacy of DNA repair mechanisms in alternate lineages; 3.) differing mutation or

fixation rates in taxa with differing life histories or generation times. Thus, a clocklike pattern of evolutionary change is an empirical issue, to be demonstrated, not postulated.

To date, the best study using DNA hybridization in birds was Sheldon's (1987) analysis of the herons (Ardeidae). About half of the total species were treated, but only 13 of the herons were included in a complete data matrix. In part this was because each pair of species was replicated 10 times. Nevertheless, the study was more complete than many biochemical studies in that all the major lineages were represented; thus the results were of real interest to systematists. However, the study indicates something of the limitations of the technique. The necessity to replicate a complete data matrix several times in order to determine the relationships of the major lineages of a moderate sized family took a considerable amount of time and effort. This study is near the upper limit of a reasonably sized investigation using DNA-DNA hybridization; it comprised Sheldon's Ph.D research.

Bledsoe's (1988) DNA-DNA study of 9-primaried oscines was also a Ph.D. project. It involved a complete 13 x 13 matrix of taxa. Of necessity, however, only a few genera of this very large assemblage could be included; thus, the results do not have immediate effect on the details of avian taxonomy. Rather, they suggest interesting problems to follow up. The same might be said of the massive data produced by Sibley & Ahlquist. Because of the widespread concerns about the details of the analysis, the lack of complete data matrices for most of the published material, and concerns about distances data in general, there is scepticism in the systematics community about the interpretation of these data (e.g. Cracraft 1987, Gill & Sheldon 1991, Lanyon 1992). It is unlikely much can be done about this, however; producing a complete data matrix for 175 avian families is not feasible with the technique. Unquestionably some of Sibley & Ahlquist's suggestions will be confirmed by future studies; but ornithologists will look to other techniques for support for these hypotheses.

Restriction enzymes

Genetic discoveries in the 1970s and 1980s made available a battery of enzymes that cleave specific sequences of DNA; for example, the restriction enzyme EcoR1 only cuts the sequence GAATTC. In a large sequence of DNA, such 'recognition sites' will occur at a particular position in some taxa and, due to evolutionary changes, not in others. With a large enough number of these enzymes and a sufficiently long sequence of DNA, a large number of character states—the cleaved sites—will be available for analysis.

An appreciable amount of research using this technique has been underway for only about 5 years. In practice, most workers have used mitochondrial DNA in order to reduce the number of sites to a reasonable number (nuclear DNA is so extensive that a large number of recognition sites invariably are found; the resulting number of fragments visualized on a gel is so great that homology becomes a problem). Analyzed cladistically, such data, if sufficient in number, can yield fully resolved branching diagrams for problems of taxonomic interest. Relatively expensive, the

technique has been used only in a few labs; it is so recent that much of the work to date has consisted of exploratory studies aimed at determining the usefulness of the method at various taxonomic levels (e.g. Avise & Zink 1988). A major impact on avian systematics is only beginning to be felt (e.g. Zink & Dittmann 1991, Gill & Slikas 1992). However, the technique is already being superseded by a newly available method for direct sequencing of DNA.

DNA sequences

Until a few years ago, actual sequencing of DNA was an expensive, elaborate procedure involving genetic cloning in vectors, etc. However, the development of the polymerase chain reaction (PCR) for gene amplification and rapid and efficient sequencing technology has now made it possible to produce large quantities of specific sequences of DNA from relatively large numbers of individuals in a reasonable period of time. Thus, it is already feasible to perform a phylogenetic study involving the DNA sequence of more than 900 base pairs for more than 20 taxa (e.g. Richman & Price 1992). If even 10% or 20% of such bases are variable, then an appreciable amount of information will be available for phylogenetic inference. Because each new sequence can be compared with all previous ones, for the same gene, the amount of work required in a study increases only as the number of species, N. The potential of this technique is such that it is rapidly being adopted by avian systematists throughout the world.

At present only a few genes are being sequenced; the most widely used is the mitochondrial cytochrome-b gene. Various domains of this gene appear to evolve at different rates—reflecting the functional constraints of alternate portions of the translated protein. The gene has already proved useful for systematics and ecological problems; for example, using cytochrome-b sequences, Richman & Price (1992) were able to produce a fully resolved (completely dichotomous tree) phylogeny for 22 species of sylviid warblers. They then used the tree to interpret patterns of morphological variation among 8 species of *Phylloscopus* warblers sympatric in the Himalayas.

Various other genes, mitochondrial and nuclear, are known to have greater (D-loop, ATPase8) and lesser (cytochrome-c oxidase) rates of evolution than cytochrome-b (Arctander 1991). It is feasible to choose such alternate genes, with their varying rates of nucleotide substitution, as a function of the taxonomic problem of interest. Thus, the technology is essentially available today to produce voluminous data on avian relationships at many levels—from populations within species, to species within genera, to families within orders (e.g. Birt-Friesen *et al.* 1992, Johnson & Cicero 1991, Edwards *et al.* 1991). The number of characters potentially available is not likely to be limiting except for vanishingly short branches. Numerical algorithms to deal with the quantity of data in a phylogenetic manner are more of a problem, but computer power is rapidly improving. It does not take great bravery to predict that a decade from now many of the major problems of avian systematics, and certainly the relationships of the families, will be solved using this technique.

Conclusions

The past century has seen a growing amount of research in the higher level systematics of birds using biochemical techniques, but particularly, and rapidly, over the past 30 years. Up to the present time, however, the results have not lived up to expectations, and it is only in recent years that a sufficient understanding of the various problems have emerged. In particular, results have been limited due to an absence of analytical method, a lack of sufficient numbers of characters, and, more recently, a failure to work on genuine systematic problems.

In Table 1, the efficacies of 8 major biochemical techniques are evaluated in terms of 3 typical problems in avian systematics. I have taken as the basis for comparison the doctoral dissertation project as the unit of time; this is convenient as many results have been obtained through such projects, providing a natural standardization; expense might have been an alternative. The simplest problem considered is the allocation of a difficult species to a family of birds; for example, what are the affinities of the Hoatzin, *Opisthocomus*? Second, what is the phylogeny of the species in a large genus or a small family, e.g. the Alcidae? Third, is the dominant problem of avian higher level systematics the relationships among avian families? Thus, for example, electrophoresis with general protein staining, intensively used in the 1960s, yielded too few characters and pre-dated quantitative analytical methods of phylogenetic inference; in retrospect this and other techniques were inappropriate for resolving many of the problems on which they were used. This same limitation was more or less true of allozyme work. Microcomplement fixation, an immunological technique, and amino acid sequencing were the first techniques available that were at all appropriate for the highest level avian systematics, but both had real limitations. Amino acid sequences were too time consuming and expensive to obtain. The immunological technique, while useful at the level of intrafamilial relationships, has N^2 complexity. Consequently, a problem involving 175 species is 7^2 times as difficult as a problem with 25 species. The recently used technique of DNA-DNA hybridization has the same complexity.

During the 1970s and 1980s, many workers in avian systematics shifted away from macrotaxonomy questions to the study of microevolutionary processes; in part this may have been due to the failure or expense of previous techniques. However, the long sought goal of molecular systematics, DNA sequences, has become feasible in the past 5 years. The technique offers access to numerous characters useful at all taxonomic levels, and algorithms for data analysis are becoming widely available. For the first time (Table 1), a technique is available that is appropriate and economical for solving the major problem in avian systematics, the relationships of the families of birds.

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